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The [NiFeSe] hydrogenase from *Desulfovibrio vulgaris* Hildenborough is a bacterial lipoprotein lacking a typical lipoprotein signal peptide

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Abstract ***Desulfovibrio vulgaris*** **Hildenborough has a membrane-bound [NiFeSe] hydrogenase whose mode of membrane association was unknown since it is constituted by two hydrophilic subunits. This work shows that this hydrogenase is a bacterial lipoprotein bound to the membrane by lipidic groups found at the N-terminus of the large subunit, which is unusual since it is missing the typical lipoprotein signal peptide. Nevertheless, the large subunit has a conserved four residue lipobox and its synthesis is sensitive to the signal peptidase II inhibitor globomycin. The *D. vulgaris* [NiFeSe] hydrogenase is the first example of a bacterial lipoprotein translocated through the Tat pathway.**

Keywords: Lipoprotein; Hydrogenase; Tat pathway; Signal peptidase II; *Desulfovibrio*

1. Introduction

The [NiFeSe] hydrogenase (HysAB) of *Desulfovibrio vulgaris* Hildenborough is very interesting from a biotechnological point of view since it has a high activity and is resistant to oxygen inactivation [1]. This hydrogenase (Hase) is preferentially synthesized by *D. vulgaris* when selenium is available [2]. It is strongly associated with the membrane, despite the fact that its two subunits are hydrophilic and it lacks a membrane cytochrome *b* subunit, present in most periplasmic uptake Hases [3]. The membrane-associated [NiFeSe] Hase ($[NiFeSe]_m$) precipitates in the absence of detergent and its activity is maximal in the presence of phospholipids. A soluble form of the [NiFeSe] Hase ($[NiFeSe]_s$) can be detected in cell extracts, and is also produced by degradation of the native $[NiFeSe]_m$ enzyme. The $[NiFeSe]_s$ form has a much reduced activity that is not affected by detergent or phospholipids. Its large subunit is missing the first 11 residues, whereas the $[NiFeSe]_m$ Hase HysA has a blocked N-terminus and a higher molecular mass than predicted from the sequence. To explain these observations it was proposed that a lipidic group present at the N-terminus of the $[NiFeSe]_m$ HysA subunit is responsible for its membrane attachment [1]. The most obvious explanation would be that the $[NiFeSe]_m$ Hase is a bacterial lipoprotein where an N-terminal Cys residue is post-translationally modified (*N*-acyl-*S*-diacylglyceryl-Cys) [4–7]. However, the sequence of HysA does not include the characteristic signal peptide used to identify bacterial lipoproteins. This signal peptide, which is cleaved by signal peptidase II (Lsp), is quite similar to those of other secreted proteins that are cleaved by signal peptidase I [8]. It includes also a positively charged n-region and a hydrophobic h-region, and its most distinguishable characteristic is a c-region consisting of four residues with the consensus [LVI][AST-VI][GAS]C, also named a lipobox, where the cleavage site occurs before the strictly conserved Cys that is the lipid modified residue [4,5]. The post-translational lipid modification involves three enzymes that act sequentially: the prolipoprotein diacylglyceryl transferase (Lgt) transfers a diacylglyceride to the cysteine sulfydryl group; the signal peptidase II (Lsp) cleaves the signal peptide at the residue before the cysteine forming an apolipoprotein; the apolipoprotein *N*-acyltransferase (Lnt) acylates the α-amino group of the apolipoprotein N-terminal cysteine forming the mature lipoprotein [5,7]. All lipoproteins described to date are translocated across the cytoplasmic membrane by the Sec pathway that transports unfolded proteins [9]. In this work, we describe experiments that confirm that the *D. vulgaris* [NiFeSe] Hase is a bacterial lipoprotein, which is translocated by the Tat pathway.

2. Materials and methods

2.1. Cell growth and preparation of the crude extracts

D. vulgaris Hildenborough (DSM 644) was grown in pyruvate-sulfate medium C, and cell extracts prepared as described [2]. To study the effect of globomycin two 50 ml cultures were inoculated in parallel. At mid-exponential phase globomycin (kindly donated by Sankyo Co., Ltd.) in methanol was added to one of the cultures at a final concentration of 50 μg/ml, and the same volume of methanol was added to the second culture. After 1 h the cultures were centrifuged and the cells suspended in Tris–HCl 20 mM, pH 7.6, and broken by sonication. Analyses by activity-stained native gels and Western blot were performed as described [2].

2.2. Experiments with lipase

The $[NiFeSe]_m$ Hase (13 μM) was incubated with 6.66 μg of *Rhizopus* lipase (from Sigma) in 20 mM Tris–HCl pH 8 (100 μl total volume). The mixture was incubated at 37 °C and aliquots were removed at different times for HPLC and gel analysis. Control samples underwent the same treatment in the absence of lipase. For peptide analysis a 24 h reaction mixture was dried by speed vacuum and extracted with methanol for 1 h. After centrifugation the supernatant was analysed by mass spectrometry.

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2.3. HPLC analysis

HPLC analysis was performed in a Beckman Gold HPLC with a Waters Symetry300 C4 column. The eluents were 0.05% trifluoroacetic acid and 0.05% trifluoroacetic acid with 80% acetonitrile, gradient 10–100% in 30 min, flow of 1 ml/min. Detection was performed at 280 and 214 nm. For identification of eluted peaks, the samples were concentrated and sequenced using an Applied Biosystem Procise HT 491 sequencer.

2.4. Mass spectrometry methods

Mass spectra of the methanolic peptide extracts were assayed in an Applied Biosystems Voyager-DE STR MALDI-TOF spectrometer as previously described [1]. The major ion in the mass spectra was subjected to post-source decay (PSD) analysis to obtain composition information. PSD fragment ion spectra were acquired as described [10].

The HPLC method described above was used to assay lipase-treated Hase by LC–MS. These analyses were performed with a Thermo Finnigan Surveyor equipment, including an LCQ ion trap mass spectrometer equipped with an ESI source, using the following conditions for the ESI source: temperature of the heated capillary, 270 °C; electrospray voltage 4.5 kV (positive mode). Nitrogen was used as sheath and auxiliary gas at the flow rates of 80 and 20 arbitrary units, respectively. LC–MS was performed in the positive and full scan modes from *m/z* 100 to 2000.

3. Results

The genome of *D. vulgaris* has 45 genes annotated as coding for putative lipoproteins, and includes also the genes for the three enzymes involved in lipoprotein synthesis: Lgt (DVU0015), Lsp (DVU1928) and Lnt (DVU1860). Strikingly, the gene coding for the Lsp signal peptidase II is found just downstream of the locus that includes the [NiFeSe] and $[NiFe]_1$ Hase genes and some of their maturation proteins (Fig. 1). This locus includes also a gene coding for a lipase from the GDSL family (*tes*A, DVU1925). None of the subunits of the $[NiFeSe]_m$ Hase is predicted to be a lipoprotein. However, the fact that the N-terminus of HysA is blocked, and that a soluble form of the enzyme is missing the first 11 residues of HysA, strongly points to the involvement of this region in the membrane association. A sequence alignment comparing the N-terminal sequences of the large subunits of *Desulfovibrio* membrane-associated Hases, with others known to be soluble, revealed a conserved M[S/G]GC motif that is absent in the soluble enzymes and is reminiscent of a lipobox (Fig. 2), pointing to the conserved Cys as a possible site for lipid modification.

The presence of a lipase in a Hase maturation operon is unprecedented and prompted us to examine the effect of a lipase on the $[NiFeSe]_m$ Hase. For this we used a *Rhyzopus* lipase as previously described [11]. The reaction mixture containing the $[NiFeSe]_m$ Hase and lipase was analysed by HPLC at different time points. The peaks observed were collected and their N-terminal sequenced. This experiment revealed that the lipase slowly converts the large subunit of the $[NiFeSe]_m$ Hase (Fig. 3, peak a) into the large subunit of the soluble $[NiFeSe]_s$ form which starts with Gly12 (Fig. 3, peak c), whereas the small subunit (peak b) remained unchanged. In a control experiment without the lipase the two subunits of the $[NiFeSe]_m$ Hase were not affected after the same incubation time. The reaction mixture was also analysed directly by LC–MS, and by native gel stained for Hase activity (Fig. 4), confirming the previous results. This experiment indicates that treatment with the lipase provokes proteolytic cleavage before Gly12 of the $[NiFeSe]_m$ Hase HysA subunit. This unexpected result may be explained by contamination of the lipase with proteases or by the actual lipase acting slowly as a protease. The lipase reaction (hydrolysis of an ester bond) is quite similar to that of a protease (hydrolysis of an amide bond) and many lipases employ a similar mechanism [12]. The reaction observed was very slow (~24 h), which agrees with a non-specific nature of the protease reaction. On the other hand, the peptidic bond between Ala11 and Gly12 is particularly susceptible to hydrolysis as the conversion of the $[NiFeSe]_m$ form into the $[NiFeSe]_s$ form can occur spontaneously with manipulation of the pure enzyme [1].

The peptide cleaved by treatment with the lipase was extracted and analysed by mass spectrometry. This revealed a prominent *m/z* peak with a value of 1601. This mass agrees with an eight residue peptide corresponding to Cys4 to

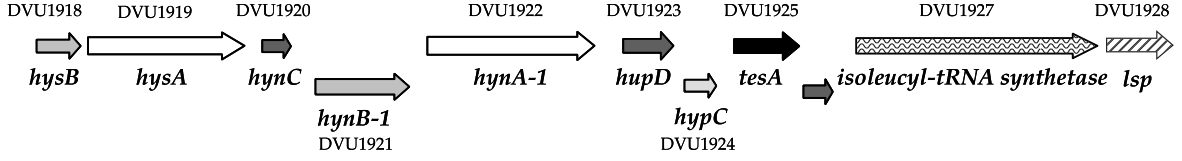

Fig. 1. The *D. vulgaris* gene locus containing the Hase genes. *hysAB*, [NiFeSe] Hase; *hynAB1*, $[NiFe]_1$ Hase; *hynC*/*hupD*, C-terminal peptidases; *hypC*, maturation chaperone; *tesA*, lipase; *lsp*, signal peptidase II.

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                          *         20        *
DvHSe       : MSGCTPKAAPAG--------ATGRTTIAIDPVTR- : 26
DdG20Se     : MSGCTNKMAAGG--------VSGKTKIAIDPVTR- : 26
DvHNiFe1    : MGGCKAKTAP-GVPVTPQSSYTG--PITIDPVTR- : 31
DvMNiFe     : MSGCRAQNAPGGIPVTPKSSYSG--PIVVDPVTR- : 32
DdG20NiFe1  : MSGCRAKTAPAGIPVTPKSDYSG--PIVVDPITR- : 32
DfNiFe      : ----------AESKP-TPQSTFTG--PIVVDPITR- : 22
Dd27kNiFe   : ----------MSQVTKTPRSNYTG--PIVVDPLTR- : 23
DgigasNiFe  : --------------------SEMQG-NKIVVDPITR- : 15
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Fig. 2. Sequence alignment of the large subunit N-terminus of several *Desulfovibrionacae* Hases. Membrane-associated Hases: DvHSe, *D. vulgaris* [NiFeSe] Hase; DdG20Se, *D. desulfuricans* G20 [NiFeSe] Hase; DvHNiFe1, *D. vulgaris* $[NiFe]_1$ Hase; DvMNiFe, *D. vulgaris* Myiazaki [NiFe] Hase; DdG20NiFe1, *D. desulfuricans* G20 $[NiFe]_1$ Hase; Soluble Hases: DfNiFe, *D. fructosovorans* [NiFe] Hase; Dd27kNiFe, *D. desulfuricans* ATTC 27774 [NiFe] Hase; DgigasNiFe, *D. gigas* [NiFe] Hase.

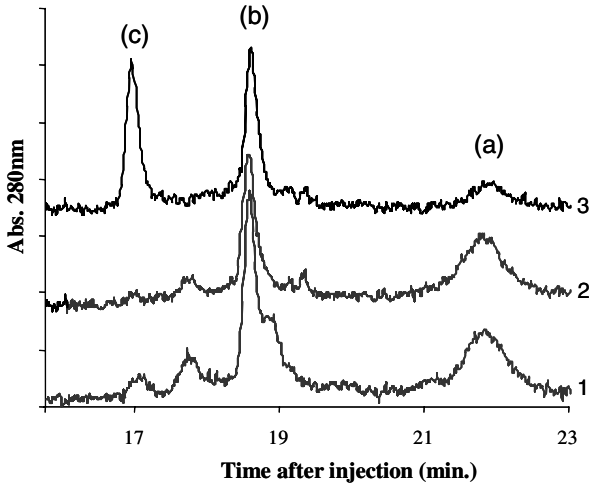

Fig. 3. HPLC analysis of the reaction between *D. vulgaris* $[NiFeSe]_m$ Hase and *Rhizopus* lipase. Trace 1: reaction mixture of Hase with lipase at time 0; Trace 2: reaction mixture containing only Hase after a 24 h incubation without lipase; Trace 3: reaction mixture containing Hase and lipase after a 24 h incubation. Peaks (b) and (c) were identified by N-terminal analysis as being HysB (GTLTG), and the HysA subunit of the $[NiFeSe]_s$ (GATGR), respectively. Peak (a) has a blocked N-terminal, which together with the fact that it is being consumed indicates that it corresponds to the $[NiFeSe]_m$ HysA subunit.

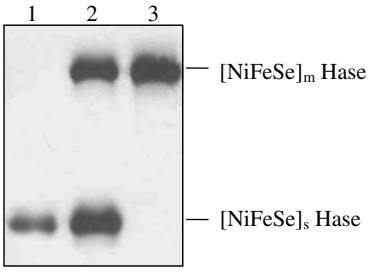

Fig. 4. Native gel stained for hydrogenase activity: (1) purified $[NiFeSe]_s$ Hase; (2) $[NiFeSe]_m$ Hase incubated for 24 h with lipase; (3) $[NiFeSe]_m$ Hase incubated for 24 h without lipase.

Ala11 (CTPKAAPA), in which the α-amino group of Cys4 is acylated, and its sulphydryl is linked to a diacylglyceride, considering that the three acyl groups correspond to two stearic (C18:0) and one palmitic (C16:0) groups. The MS/MS spectrum of *m/z* 1601 shows a major peak at 1037. The mass difference of 565 units can be explained by the loss of the diacylglyceride part, containing a stearic and a palmitic group.

We also examined the effect of globomycin, a cyclic peptide that is a specific inhibitor of signal peptidase II [13,14]. Analysis of cell extracts by native gels using Western blot and activity staining revealed that in cells treated with globomycin a second band appears (Fig. 5), corresponding to the prolipoprotein form of the Hase that accumulates as a result of blockage of the second step of the lipoprotein maturation. The two forms could not be distinguished by SDS–PAGE, which would produce better defined bands, since the mass difference in HysA is very small, as the prolipoprotein will only have three more residues (MSG) and one less acyl group than the mature protein. In the control experiment where globomycin was not added, the prolipoprotein band was not detected.

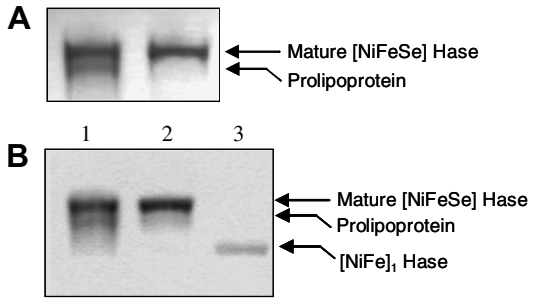

Fig. 5. (A) Western blot on native gel of *D. vulgaris* crude extracts with an anti-[NiFeSe] Hase antibody: (1) cells treated with globomycin; (2) cells not treated with globomycin. (B) Native gel stained for Hase activity of the same samples and (3) *D. vulgaris* $[NiFe]_1$ Hase as a control.

4. Discussion

Most uptake [NiFe] Hases are found in the periplasm bound to the cytoplasmic membrane by a cytochrome *b* subunit that is involved in electron transfer with the menaquinone pool, and a hydrophobic region found in the C-terminus of the small subunit. In [NiFe] and [NiFeSe] Hases from *Desulfovibrio* spp. both the cytochrome *b* and the membrane anchor of the small subunit are not present, and the electron acceptor is the soluble Type I cytochrome c_3 [15]. Nevertheless, several Hases from these organisms have been reported to be membrane-associated [16], despite being constituted by hydrophilic subunits. This is the case of the $[NiFe]_1$ and [NiFeSe] Hases from *D. vulgaris*, both of which have a large subunit with a blocked N-terminus [1,17]. The maturation of [NiFe] Hases is a complex process that occurs in the bacterial cytoplasm [3]. The periplasmic uptake Hases are thus translocated across the membrane by the Tat transport system for folded proteins [18]. The twin-arginine signal peptide is present only in the small subunit, and it has been demonstrated that the catalytic subunit is co-translocated in a complex with the small subunit [19].

Bacterial lipoproteins described to date are translocated by the Sec pathway, and their signal peptide is cut by signal peptidase II, which is specific for lipoproteins, whereas signal peptidase I cuts signal peptides of both Sec and Tat translocated proteins. However, some predicted lipoproteins contain twin-arginine signal peptides suggesting that lipoprotein transport by the Tat pathway may also be possible [20], but has never been demonstrated. The results described here confirm that the [NiFeSe] Hase is a bacterial lipoprotein, bound to the membrane by three acyl groups attached to the modified Cys4 of HysA. This protein is the first example of a lipoprotein where the signal peptide is limited to the three residues of the lipobox. The other two regions of lipoprotein signal peptides are involved in recognition and transport by the Sec pathway. In the case of HysA they are unnecessary as this protein is translocated by the Tat pathway in complex with HysB, which contains a Tat signal peptide. After translocation, the signal peptide of HysB is cleaved by signal peptidase I, and the prolipoprotein form of HysA with a diacylglyceride bound to Cys4 is cleaved by peptidase II, removing the first three residues. The results permit two interesting conclusions: the first is that lipoprotein synthesis may occur in the absence of a standard signal peptide with only the lipobox being necessary for correct lipoprotein processing by the signal peptidase II; the

second is that such processing may occur with a folded protein, translocated by the Tat system rather than the usual Sec system.

In structural terms the N-terminus of HysA is in the opposite side of this protein relative to the HysB subunit, which indicates that the membrane attachment by the lipidic group does not interfere with electron transfer between the hydrogenase (through HysB) and its electron acceptor, the Type I cytochrome c_3 [1]. It is noteworthy that a significant proportion of this cytochrome is also found associated with the membrane [15]. The conserved lipobox in the large subunit of several other *Desulfovibrionacae* Hases, including the *D. vulgaris* $[NiFe]_1$ (but not $[NiFe]_2$ Hase) points to lipid modification also in these cases. The membrane attachment of these Hases may be advantageous as the electrons from H_2 oxidation will be transferred to membrane-bound redox complexes through the Type I cytochrome c_3 [15].

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